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Toxicology 171 (2002) 117–125

TOXICOLOGY

www.elsevier.com/locate/toxicol

The effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on oxidative enzymes in adipocytes and liver

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Received 23 February 2001; received in revised form 25 October 2001; accepted 31 October 2001

Abstract

Reactive oxygen species are produced in response to environmental toxins, and previous studies have suggested that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) damages a number of target organs through the generation of oxygen free radicals and oxidative stress. Upon exposure, TCDD becomes concentrated in adipose tissue, and adversely affects many organs, including liver. This study examined whether oxidative stress was induced in adipocytes and liver that were exposed to TCDD. 3T3-F442A adipocyte cultures were treated with TCDD (5–200 nM) for up to 72 h, and the activity and mRNA levels of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) in adipocyte cell lysates were measured. The addition of 50 nM TCDD induced a two-fold increase in SOD activity after 48 h ($P < 0.05$). In contrast, TCDD had no significant effect on the activity of catalase or GSH-Px in the adipocytes, and the increase in SOD activity was not accompanied by a change in SOD mRNA levels. To assess the effects of TCDD on oxidative stress enzymes in vivo, male Sprague–Dawley rats were injected weekly for 8 weeks with 30 ng/kg TCDD. In addition, the rats were fed either a low-fat complex-carbohydrate (LFCC) diet, or a high fat sucrose diet (HFS). The HFS diet has previously been shown to induce mild obesity and insulin resistance, without inducing diabetes. SOD, catalase, and GSH-Px activities were measured in the liver and adipose tissue of these rats. TCDD injection resulted in a 52% decrease in catalase activity in the adipose tissue of HFS rats ($P < 0.05$). In contrast, SOD and GSH-Px activities were not altered in adipose tissue of TCDD-injected rats. In liver, however, there were significant decreases in GSH-Px activity in response to TCDD. This effect of TCDD was observed in both the LFCC and HFS dietary groups. In addition, GSH-Px activity in the HFS rats was significantly decreased when compared to GSH-Px activity in LFCC rats, in both TCDD-treated and control groups, suggesting that TCDD and a high fat diet may combine to exacerbate oxidative stress. Thus, TCDD induces complex changes in enzymes of oxidative stress in both adipocytes and liver. In adipocytes, these changes occurred post-transcriptionally, as there were no changes in mRNA levels. In addition, a high fat diet per se also resulted in a decrease in GSH-Px activity in liver.

Keywords: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Oxidative enzymes; Adipocytes and liver; High fat diet

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0300-483X/02/$ - see front matter
PII: S0300-483X(01)00564-9

1. Introduction

The generation of reactive oxygen species (ROS) can lead to oxidative stress, cell damage, and disease. A hallmark of oxidative stress is lipid peroxidation, which disrupts the structural integrity of cell membranes and can also lead to the formation of aldehydes, which in turn further damage lipids, protein, and DNA. Cells possess defense mechanisms to protect against free radical damage including enzymes such as superoxide dismutase (SOD), which scavenge free radicals to form non-radical products. Two other key 'antioxidant' enzymes are catalase and glutathione peroxidase (GSH-Px), both of which decompose peroxides.

ROS are produced in response to exposure to environmental toxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Stohs, 1990). TCDD is a member of the polychlorinated dibenzo-*p*-dioxin family and is a toxic contaminant of herbicides such as 'Agent Orange', which was used extensively during the Vietnam war (Kahn et al., 1988). In experimental animals, TCDD exposure damaged a number of target organs including liver, thymus, and adipose tissue (Poland and Knutson, 1982). TCDD exerts many of its effects by binding to the Ah receptor (Landers and Bunce, 1991; Nebert et al., 2000; Okey et al., 1994) and inducing cytochrome P-450 gene expression (Whitlock, 1989). One by-product of increased cytochrome P450 activity is increased incidence of electron transfer to molecular oxygen leading to ROS formation and lipid peroxidation (Goeptar et al., 1995). A number of studies have administered TCDD to rodents, and observed changes associated with increased oxidative stress, including increased superoxide formation (Bagchi and Stohs, 1993), lipid peroxidation (Stohs, 1990), and DNA single strand breaks (Shertzer et al., 1998; Wahba et al., 1988). In addition, another study (Slezak et al., 2000) demonstrated that indices of oxidative stress were present after both acute and subchronic administration of TCDD to mice, but the ROS production required only low tissue levels of TCDD in the subchronic exposure mice.

Upon exposure, TCDD is concentrated in adipose tissue, and several studies have drawn an epidemiologic link between previous TCDD exposure and the development of diabetes (Henriksen et al., 1997; Longnecker and Michalek, 2000). A recent study has also suggested that non-diabetic subjects with high blood dioxin levels demonstrate insulin resistance (Cranmer et al., 2000). In addition to the links between TCDD exposure and the development of diabetes and/or insulin resistance, there is considerable evidence that diabetes and diabetes-related pathologies are associated with oxidative stress (Baynes, 1991; Giugliano et al., 1996; Wolff, 1993). In rats with streptozotocin-induced diabetes, catalase, SOD, and glutathione reductase were increased in the pancreas, whereas GSH-Px, SOD, and catalase were decreased in the liver (Wohaieb and Godin, 1987). In most cases, insulin treatment restored antioxidant enzyme activity levels to values comparable to those of non-diabetic controls. Oxidative stress may have contributed to the development of diabetes since streptozotocin-induced hyperglycemia was attenuated in transgenic mice overexpressing SOD (Kubisch et al., 1994). In addition, in patients with diabetes, there was increased lipid peroxidation in erythrocytes coincident with decreased GSH-Px activity (Uzel et al., 1987). Other studies of antioxidant enzyme levels in diabetic patients showed altered erythrocyte SOD concentrations, SOD activity, and glutathione reductase activity (Collier et al., 1990; Godin et al., 1988). The mechanisms whereby oxidative stress is invoked in diabetes are not well understood and are probably complex. One possible mechanism for hyperglycemia-mediated oxidative stress involves the autoxidation ability of glucose, which can result in the production of superoxide radicals and other ROS (Hunt et al., 1990).

Because adipose tissue is both a target of TCDD action, and an important organ in the development of diabetes, we wished to determine whether TCDD induced oxidative stress in adipocytes. In addition, this study examined whether TCDD would induce oxidative stress in rats that were already insulin resistant from the feeding of a high fat diet.

2. Methods

2.1. Cells and tissue culture

3T3-F442a cells were obtained from Dr Howard Green (Harvard Medical School, Boston, MA) (Green and Meuth, 1974), and were cultured in 75 cm^2 flasks in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% calf serum and penicillin and streptomycin. For experiments, cells were subcultured in 12-well dishes. Confluent cultures were allowed to differentiate by adding DMEM containing 10% fetal bovine serum and insulin (1 μg/ml), isobutylmethylxanthine (0.5 mM), and dexamethasone (0.25 μM) for 72 h. Cells were then maintained in DMEM containing 10% serum and 1 μg/ml insulin for 5–7 days. Medium was then changed to DMEM containing 10% serum, and cells were used for the experiments described. TCDD was obtained from Cambridge Isotopes (Andover, MA), and the purity was 98% as verified by analysis (Radian International, Austin, TX). TCDD dissolved in DMSO was added to culture medium to a final concentration of either 5, 50, or 200 nM for 24, 48, or 72 h. For each experiment, control cultures received DMSO alone at the same concentration.

2.2. Animals and diets

Male Fisher rats (Harlan Sprague–Dawley) weighing 200 g were stabilized on standard laboratory chow for 5 days, and the animals were then divided into four groups of eight rats each. Half the rats were fed the low-fat complex-carbohydrate (LFCC) diet and the others were fed a high-fat sucrose (HFS) diet, as described previously (Barnard et al., 1993). The percentage of calories in protein, fat and carbohydrate were 21, 6, and 73, respectively, for the LFCC and 21, 39.5 and 39.5, respectively, for the HFS. The diets were prepared in powdered form by Purina Test Diets with vitamin-free casein as the source of protein. The source of fat in the HFS diet group consisted of 90% lard and 10% corn oil. Both diets contained a standard mineral and vitamin mix. Food and water were given ad libitum on 12 h light and dark cycles. Half the rats in each diet group were injected weekly IP with 30 ng/kg TCDD, while the others were injected with corn oil carrier. This protocol was carried out for 8 weeks, after which the animals were sacrificed. The weekly TCDD injection scheme was used to avoid TCDD-mediated toxicity, to prevent anorexia, and to mimic the chronic exposure characteristic of humans. There was no perceptible change in food intake or weight gain as a result of TCDD when the rats were given free access to food. After 8 weeks, the weights of the animals were as shown in Table 1. The HFS rats were slightly heavier than the LFCC rats, but there was no effect of TCDD on animal weight or blood glucose. Upon sacrifice, the liver and epididymal fat pads were immediately removed and frozen at -80 °C until activity assays were performed for GSH-Px, SOD, and catalase.

2.3. Measurements and assays

The data for all enzyme assays were expressed per mg cell protein (BioRad reagent, Richmond, CA), where bovine serum albumin was the protein standard. Cells or tissues (rat adipose tissue or liver) were homogenized in buffer solutions, as described below, centrifuged at $5000 \times g$ for 15 min, and the supernatant fractions were used for the assay.

Table 1
Effect of TCDD and diet on body weight and blood glucose in rats

	LFCC	LFCC+TCDD	HFS	HFS+TCDD
Weight (g)	339 ± 4	339 ± 6	358 ± 8*	373 ± 9*
Glucose (mg/dl)	166 ± 6	169 ± 6	158 ± 7	162 ± 11

*$P < 0.05$ vs. LFCC.

SOD was measured using a SOD activity assay kit (CALBIOCHEM, Catalog number 574600). 3T3-F442A cells or tissues were harvested and cell lysates were prepared according to kit specifications. This assay utilized a reagent (5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene) which undergoes an accelerated autoxidation in the presence of SOD. This autoxidation yields a chromophore which absorbs maximally at 525 nm. The kinetic measurement of the 525 nm absorbency change is performed after the addition of reagent and SOD activity is determined from the ratio of the autoxidation rates in the presence and in the absence of SOD. One unit of SOD activity is defined as the activity that doubles the autoxidation rate of the control blank. Enzyme activity was calculated based on the slope of the reaction rate within the linear region of the plot.

GSH-Px activity was measured using a cellular GSH-Px activity kit (CALBIOCHEM, Cat. No. 354104). Cells or tissues (rat adipose tissue or liver) were homogenized in 4–8 ml of cold buffer (50 nM Tris–HCl, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol) and the supernatant fractions were collected by centrifugation at $5000 \times g$ for 15 min. Samples containing GSH-Px were added to a solution containing GSH, glutathione reductase, and NADPH, and the rate of decrease in absorbance at 340 nm, due to the oxidation of NADPH, was measured. The rate of oxidation of NADPH to $NADP^+$ is directly proportional to GSH-Px activity. Enzyme activity was calculated based on the slope of the reaction rate within the linear region of the plot.

Catalase activity was measured as described previously (Aebi, 1984). This assay involves the change in absorbancy at 240 nm due to the catalase dependent decomposition of H_2O_2. Cells were lysed by freeze/thaw three times, and tissues were homogenized in PBS. The supernatant fractions were collected after centrifugation and H_2O_2 was added to each sample in a starting concentration of 8.8 mM. The change in absorbance at 240 nm was measured for 30 s and the slope of the curve at linearity was calculated. Data are expressed as μmol of peroxide decomposed/s/mg protein.

2.4. Northern blotting

Northern blotting was performed using RNA isolated from the cells and tissues using guanidine thiocyanate as described (Chomczynski and Sacchi, 1987). Equal amounts of RNA were electrophoresed on a 1% agarose–formaldehyde gel, transferred to nylon membrane (Nylon 1, GIBCO.BRL) and hybridized with [^{32}P]-cDNA probes for mouse SOD, GSH-Px, and catalase (American Type Culture Collection). The blots were probed for glyceraldehyde phosphate dehydrogenase (GAPD) (Tso et al., 1985) and γ-actin (Gunning et al., 1983), to control the amount of RNA loaded in the gel. Northern blots were then scanned using densitometry to permit comparisons between different treatments.

2.5. Statistics

All data were expressed as the mean ± SEM, and were analyzed using the Student's *t*-test. To analyze data between multiple groups, ANOVA was performed, followed by secondary analysis using the Student's *t*-test with Bonferroni correction.

3. Results

To examine the effects of TCDD on adipocyte oxidative stress, TCDD was added to cultures of 3T3-F442A adipocytes, as described in Section 2. TCDD was added in concentrations of 5, 50, and 200 nM, and the enzymes of oxidative stress (GSH-Px, SOD, and catalase) were measured. Fig. 1 shows the effect of 50 nM TCDD on the activity of SOD, catalase and GSH-Px at 48 h. There was a significant increase in SOD activity. This increase in SOD activity was not observed at 24 h after the addition of TCDD, as shown in Fig. 2. To examine SOD mRNA levels, Northern blotting was performed. As shown in the representative Northern blot in Fig. 3, there was no increase in SOD mRNA level, and this observation was verified by densitometry analysis of additional Northern blots from cells treated with the same concentrations of TCDD. Thus, this in-

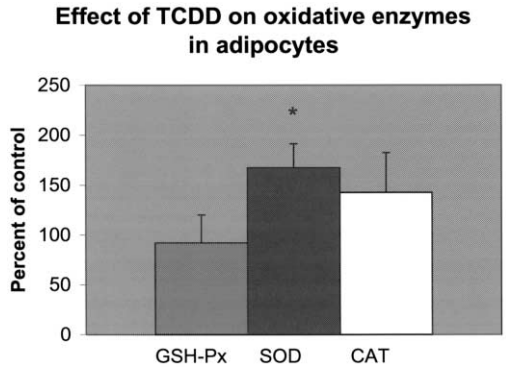

Fig. 1. Effect of TCDD on GSH-Px, SOD, and catalase activities. TCDD was added to 3T3-F442a cells in a concentration of 50 nM for 48 h. Control cultures contained DMSO (vehicle for TCDD), and data are expressed as percent of control. $*P < 0.05$ vs. control cells.

crease in SOD activity in the absence of an increase in SOD mRNA levels suggested post-transcriptional mechanisms of regulation. Catalase activity increased in a dose–response manner, with an increase at 48 h with the addition of 200 nM TCDD (Fig. 4), however, these changes were not statistically significant. There were no changes in the activity of GSH-Px in 3T3-F442A adipocytes in response to TCDD, even after 72 h in culture and the addition of up to 200 nM TCDD (data not shown). In addition, there were

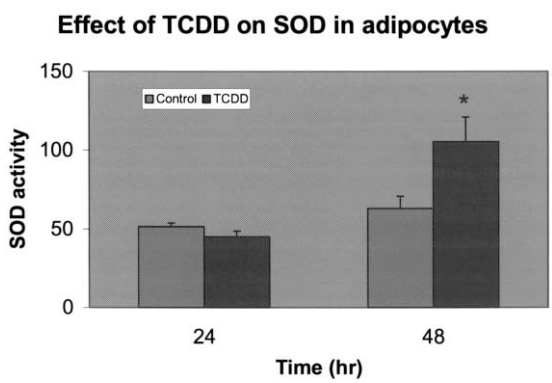

Fig. 2. Effect of TCDD on SOD activity. TCDD (50 nM) was added to 3T3-F442a adipocyte cultures and SOD activity was measured as described in Section 2 at 24 and 48 h. SOD activity was expressed as units/mg protein. $*P < 0.05$ vs. control.

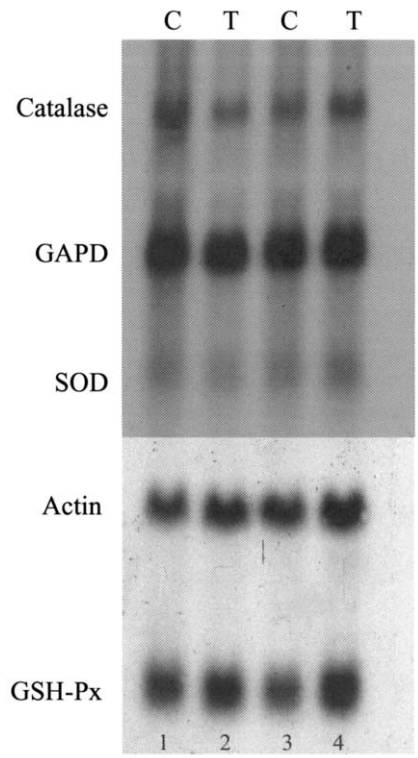

Fig. 3. Effect of TCDD on oxidative enzyme mRNA levels. TCDD was added to 3T3-F442a adipocytes in a concentration of 10 (lane 2) or 50 nM (lane 4) followed by RNA extraction and Northern blotting, as described in Section 2. Shown is a representative Northern blot that was probed initially for catalase, SOD, and GAPD (a constitutive probe), and subsequently for GSH-Px and γ-actin. This and other Northern blots were scanned using densitometry for a quantitative analysis. Overall, TCDD had no effects on the mRNA levels of catalase, SOD, or GSH-Px. C, control; T, TCDD.

no changes in the mRNA levels of catalase or GSH-Px (Fig. 3).

To determine whether TCDD alters oxidative enzyme activity in vivo, rats were injected with 30 ng/kg TCDD weekly for 8 weeks. As described in Section 2, the rats were also placed on either a LFCC diet, or a HFS diet. Rats were sacrificed and adipose tissue and liver were removed for analysis of oxidative enzyme activity. As shown in Fig. 5, weekly TCDD injections resulted in a significant 52% decrease in catalase activity in adipose tissue. Adipose tissue demonstrated no changes in GSH-Px or SOD, and there was no specific effect of the diet on any of the enzymes.

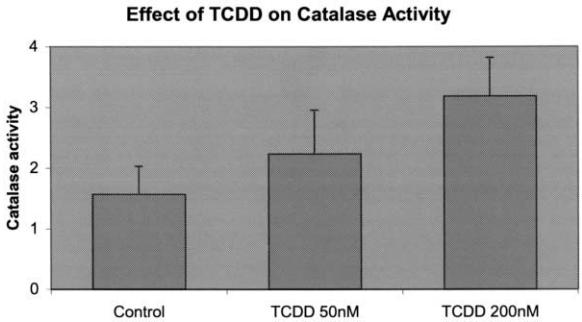

Fig. 4. Effect of TCDD on catalase activity. TCDD in the indicated concentrations were added to 3T3-F442a adipocytes for 48 h, and catalase activity was measured. Data are expressed as μmol H_2O_2/s/mg protein (Section 2).

Fig. 6 illustrates the effects of the diets and TCDD in liver. Liver GSH-Px activity was significantly decreased in response to TCDD. This effect was seen in both the LFCC and HFS diet groups. In addition, liver GSH-Px activity was decreased in the HFS diet groups relative to the LFCC diet groups.

4. Discussion

This study examined the effects of TCDD on markers of oxidative stress in adipose tissue and liver. We studied adipocytes because TCDD is highly lipid soluble, and concentrates in adipose tissue following in vivo exposure. Adipocytes were examined after the addition of TCDD in vitro, as well as after TCDD injection into rats. Because liver is the major organ involved in detoxification, and an important organ in the generation of ROS, we also examined liver tissue from rats following TCDD injection.

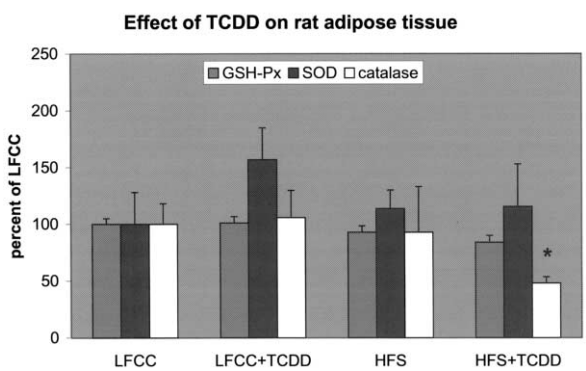

Fig. 5. Effect of weekly TCDD injections and a high fat diet on adipose tissue oxidative enzyme activity. Rats were fed either the LFCC or HFS diets, and injected with 30 ng/kg TCDD weekly, as described. GSH-Px, SOD, and catalase were measured in the adipose tissue as described. Data are expressed as a percent of the LFCC rats. $*P < 0.05$ vs. LFCC.

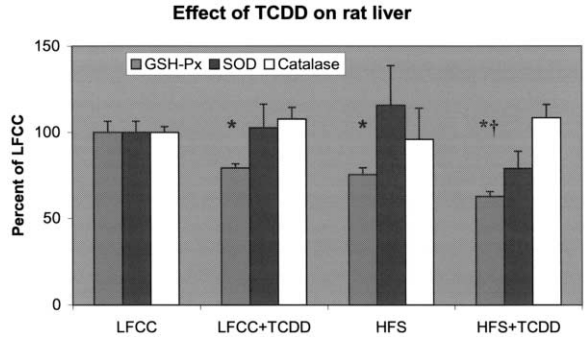

Fig. 6. Effect of weekly TCDD injections and a high fat diet on liver oxidative enzyme activity. Rats were fed either the LFCC or HFS diets, and injected with 30 ng/kg TCDD weekly, as described. GSH-Px, SOD, and catalase activities were measured in the liver as described. Data are expressed as a percent of the LFCC rats. $*P < 0.05$ vs. LFCC. $\dagger P < 0.05$ vs. HFS.

Although the addition of TCDD to adipocytes had no effect on GSH-Px activity, there was a significant increase in SOD activity, and a small, but insignificant increase in catalase activity. It is interesting to note that there was no change in SOD mRNA levels from the adipocytes, suggesting that the changes in SOD activity were due to post-transcriptional changes. Thus, this increase in SOD activity may provide a protective mechanism for the adipocyte against TCDD-induced oxidative stress.

Additional studies in vivo involved rats, which were treated with TCDD for 8 weeks at a dose of 30 ng/kg/week. This dose of TCDD was intentionally low (LD_{50} of TCDD in the Fisher rat is approximately 60 μg/kg), and was intended to mimic the type of repeated, low-level environmental contamination in human populations (e.g. industrial workers, Agent Orange exposure) (Michalek et al., 1995). Because we were interested in the possible role of diabetes and insulin

resistance, we made some rats relatively insulin resistant by HFS feeding. In previous studies, the HFS rats have been shown to be non-diabetic and insulin resistant, with high fasting insulin levels and normal fasting glucose levels (Barnard et al., 1995). Furthermore, this rat model has been shown in previous studies to manifest other features of the 'insulin resistance syndrome', including obesity, hypertension, hypertriglyceridemia and enhanced clotting (Barnard et al., 1993). As shown in Fig. 5, the adipose tissue of rats treated with both the HFS diet and TCDD manifested a significant decrease in catalase activity. In contrast to the decreased catalase activity we observed in vivo, 3T3-F442A adipocytes demonstrated no significant change in catalase activity, and certainly no evidence of a decrease in catalase activity, following TCDD treatment. This difference between in vivo and in vitro results could be due to a secondary effect brought on by the TCDD in vivo, or by an interaction between the HFS diet and TCDD.

One substance which may have interacted with TCDD in vivo is TNF. The adipose tissue of rodents and humans express TNF, and TNF expression increases with obesity (Hotamisligil et al., 1993; Kern et al., 1995) and high fat feeding (Uysal et al., 1997), which may explain some of the insulin resistance of obese rodents and humans (Kern, 1997). Some of the toxic effects of TCDD in cells occur through the expression of TNF (Dohr et al., 1994; Fernandez-Checa et al., 1997; Vogel and Abel, 1995). For example, administration of anti-TNFα antibody resulted in less TCDD-induced oxidative stress, as measured by DNA single strand breaks, in hepatic nuclei (Alsharif et al., 1994), and anti-TNF antibodies have also been found to reduce TCDD-mediated mortality in mice (Taylor et al., 1992). Therefore, the HFS rats may have been more susceptible to TCDD-mediated increases in TNF because of the existing high TNF expression.

An additional interaction between TCDD and TNF involves the induction of apoptosis. Both TNF and TCDD have been shown to induce an increase in apoptosis in a variety of cells. In previous studies, TCDD impaired differentiation in adipocytes (Phillips et al., 1995), and induced apoptosis in thymocytes, granulosa cells, T-cells, and embryonic cells (Cantrell et al., 1998; Heimler et al., 1998; Hossain et al., 1998; Kamath et al., 1997). The induction of apoptosis occurred through a mechanism that did not involve the Ah receptor (Hossain et al., 1998). TNF also resulted in the induction of apoptosis in a number of cells, including adipocytes (Haimovitz-Friedman et al., 1997; Prins et al., 1997). Thus, it is possible that the administration of TCDD may have combined with the increased TNF expressed in rats fed the HFS diet. Both TNF and TCDD may have led to an increase in oxidative stress (Alsharif et al., 1994; Obrador et al., 1998), which may have contributed to apoptosis and hence yielded the changes observed in catalase expression.

Another possible interaction between the HFS diet and TCDD injections involves the activity of GSH-Px in liver. As shown in Fig. 6, the HFS diet alone resulted in a decrease in GSH-Px activity, as did the injection of TCDD. TCDD resulted in lower levels of GSH-Px activity in rats fed both the LFCC and HFS diets. Previous studies have noted decreased hepatic GSH-Px in rats given TCDD (Stohs et al., 1986). In addition, rats with streptozotocin-induced diabetes demonstrated decreased GSH-Px, SOD, and catalase in the liver (Wohaieb and Godin, 1987). Therefore, previous data suggest that the oxidative stress induced by TCDD and by diabetes may combine to contribute to diabetes-related pathologies. The present study extends these findings suggesting that a HFS diet, leading to obesity and insulin resistance, may also combine with TCDD to yield an increase in oxidative stress.

Previous studies have examined the relationship between oxidative stress and diabetes and have suggested that there is considerable variability in terms of which antioxidant enzymes are altered in particular tissues, and whether they are increased or decreased. In the present study, we also found TCDD-induced alterations in enzyme activity to vary in their direction. Increased antioxidant enzyme activity would suggest a compensatory mechanism to defend against oxidative stress. In contrast, decreased antioxidant enzyme activity may be due to a depletion of antioxidant enzymes in response to oxidative stress. For example, the

finding that GSH-Px is decreased in liver following TCDD treatment may reflect diminished enzyme resources in response to oxidative stress. Another possibility is that TCDD is primarily affecting other compounds. For example, it is possible that the level of glutathione (GSH), the preferred source of reducing equivalents for GSH-Px, is diminished and that in turn leads to decreased GSH-Px activity.

In summary, the in vitro treatment of 3T3-F442a adipocytes with TCDD resulted in an increase in SOD activity, and the injection of rats with TCDD altered the levels of catalase activity in adipose tissue, and GSH-Px activity in liver. The feeding of a HFS high fat diet to the rats augmented these effects of TCDD in vivo. These changes in SOD, catalase, and GSH-Px activity suggest that these tissues were experiencing oxidative stress, which may contribute to numerous pathologies, including diabetes and/or insulin resistance.

Acknowledgements

This was supported by NIH grants ES 09346 and DK 39176, a Merit grant from the Veterans Administration, and a grant from the Arkansas affiliate of the American Heart Association. We wish to thank Sarah Dunn for clerical assistance.

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